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Enzyme-Catalysed Synthesis of Cyclohex-2-en-1-one *cis*-Diols from Substituted Phenols, Anilines and Derived 4-Hydroxycyclohex-2-en-1-ones

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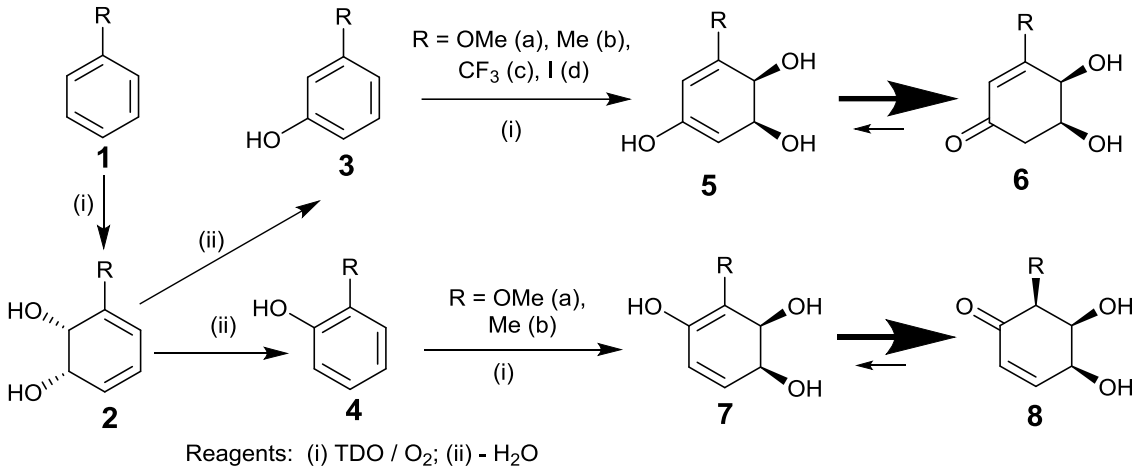
Abstract: Toluene dioxygenase-catalysed *cis*-dihydroxylations of substituted aniline and phenol substrates, with a *Pseudomonas putida* UV4 mutant strain and an *Escherichia coli* pCL-4t recombinant strain, yielded identical arene *cis*-dihydrodiols, which were isolated as the preferred cyclohex-2-en-1-one *cis*-diol tautomers. These *cis*-diol metabolites were predicted by preliminary molecular docking studie, of anilines and phenols, at the active site of toluene dioxygenase Further biotransformations of cyclohex-2-en-1-one *cis*-diol and hydroquinone metabolites, using *Pseudomonas putida* UV4 whole cells, were found to yield 4-hydroxycyclohex-2-en-1-ones as a new type of phenol bioproduct. Multistep pathways, involving ene reductase and carbonyl reductase-catalysed reactions, were proposed to account for the production of 4-hydroxycyclohex-2-en-1-one metabolites. Evidence for the phenol hydrate tautomers of 4-hydroxycyclohex-2-en-1-one metabolites was shown by formation of the corresponding trimethysilyl ether derivatives.

Keywords: aniline biotransformations; cyclohex-2-en-1-one *cis*-diols; 4-hydroxycyclohex-2-en-1-ones; phenol hydrates.

Introduction

Enzyme-catalysed formation of phenol metabolites, from aromatic substrates, can proceed directly [1a] or indirectly, *via* transient intermediates. Phenols have been obtained indirectly by: (a) monooxygenase- or peroxygenase-catalysed arene epoxidation and spontaneous isomerization of arene oxide-oxepine intermediates[1b-e] or (b) dioxygenase-catalysed *cis*-dihydroxylation of arenes[2a-m] and dehydration of *cis*-dihydrodiol intermediates.[3a,b] Since their discovery by Gibson *et al* in 1968,[2a] more than four hundred *cis*-dihydrodiol metabolites of substituted monocyclic and polycyclic arenes have been isolated and identified using, mainly, *Pseudomonas putida* mutant and *Echerichia coli* recombinant strains, expressing ring-hydroxylating dioxygenases.[2b-m] Synthetic applications of enantiopure substituted benzene *cis*-dihydrodiols **2**, obtained as toluene dioxygenase (TDO)-catalysed *cis*- dihydroxylation products, from monosubstituted benzene substrates **1**, continue to be widely reported, despite their limited stability.[2b-m] Dehydration of most *cis*-dihydrodiols **2**, to yield a mixture of *meta*- and *ortho*-phenols **3** and **4**, can occur at ambient temperature. The rate of dehydration and ratio of phenol isomers depend on the type of



Scheme 1. Tandem TDO-catalysed *cis*-dihydroxylation of monosubstituted benzenes (**1**) and phenols (**3**, **4**) to yield *cis*-dihydrodiols (**2**, **5**, **7)** and cyclohex-2-en-1-one *cis*-diol keto-tautomers (**6**, **8**).

substituent and pH of solution (Scheme 1).[3a,b]

`Since phenols are widely distributed in the environment, as natural products, arene metabolites and environmental pollutants, their biodegradation pathways have been extensively studied.[4a-g] Bacterial metabolism of phenols often results in a wider range of metabolites, compared with most non-phenolic aromatic substrates. Recent biotransformation results of TDO-catalysed oxidations of phenols, *e.g.* **3** and **4**, using *P. putida* UV4, showed that, in addition to the expected, catechol and hydroquinone metabolites, the corresponding cyclohex-2-en-1-one *cis*-diols **6a-d**, **8a** and **8b** were also isolated, as the preferred keto tautomers of the initially formed enolic *cis*-dihydrodiols **5a-d**, **7a** and **7b**. The dihydroxylation was regio- and stereo-selective and cyclohex-2-en-1-one *cis*-diols **6a-d**, **8a** and **8b** were often found to be the major isolated metabolites. More than twenty members of this new *cis*-diol family have now been isolated.[5a-e]

The biodegradation pathways, for anilines, have also been studied, due to their presence in the environment, as a result of the partial combustion of tobacco and automotive fuels, the application of pesticides / herbicides and the production of pharmaceuticals, dyestuffs and textiles.[6a-j] Many aniline derivatives are known to be genotoxic and cytotoxic, severely inhibiting cell growth in soil bacteria and slowing their mineralization.[6a,g]

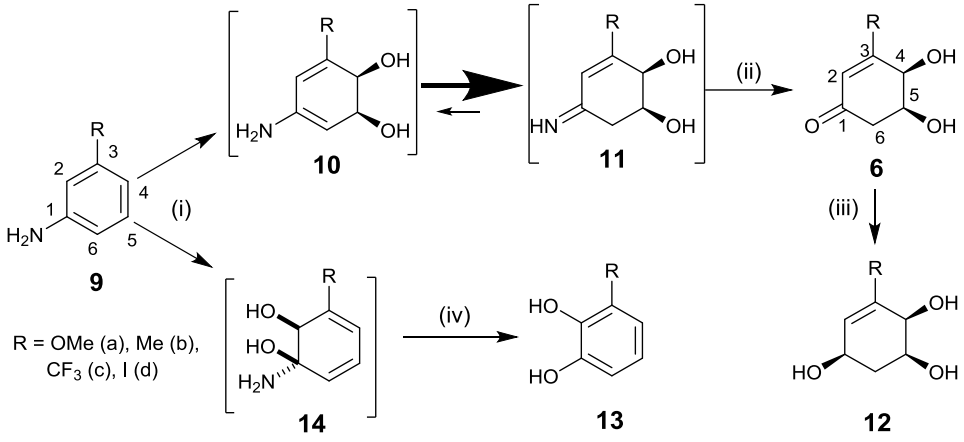
Similar to the metabolism of electron-rich phenols,[5a-e] ring hydroxylating dioxygenase-catalysed biotransformations of electron-rich anilines (using *Pseudomonads* and other bacterial species) have also been reported, to yield catechol and hydroquinone metabolites.[6a-j] Aniline-, biphenyl-diphenylamine- and toluene-dioxygenases, have been reported to catalyse the oxidation of aniline substrates.[6d, f-i] Although *cis*-diol metabolites were often postulated as intermediates leading to the formation of catechol, hydroquinone and phenol metabolites of anilines,[6c-j] to date none have been detected or isolated. Thus, TDO-catalysed *cis*-dihydroxylation, at the 1,2- and 2,3-bonds of 4-chloroaniline, was proposed (using *P. putida* T57) as a possible initial step in the formation of both catechol and phenol metabolites.[6i] Biotransformations using other substituted anilines and bacterial strains, were also found to yield catechol and hydroquinone metabolites.[6a,c] *In silico* molecular docking studies, on aniline substrates, were thus conducted to: (a) predict the most favourable structures of expected metabolites and (b) compare the substrate docking results with the experimental results of TDO-catalysed *cis*-dihydroxylation of aniline substrates.

Results and Discussion

P. putida UV4 biotransformations of aniline and phenol substrates, to yield cyclohex-2-en-1-one *cis*-diols and 4-hydroxycyclohex-2-en-1-ones

(i) Molecular docking of *meta*-substituted aniline substrates 9a-d with TDO

Recent molecular docking studies, of the *meta* substituted phenols **3a** and **3b**, at the active site of TDO,[5f] were based on a comparison with an X-ray crystal structure of TDO and docked toluene substrate.[5g] These studies of TDO, without dioxygen incorporation (3EN1M model), provided preferred orientations of phenol substrates **3a** and **3b** (Scheme



Scheme 2. TDO-catalysed *cis*-dihydroxylation of *meta*-anilines **9** to yield *cis*-diols **6**, *cis*-triols **12** and catechols **13.**

1) required for production of the corresponding enol *cis*-dihydrodiols **5a** (12% docking iterations) and **5b** (66% docking iterations). Catechols **13a** and **13b** (Scheme 2) were also predicted (54% docking iterations from phenol **3a** and 34% from **3b**).

Docking of phenols **3a** and **3b**, with dioxygen incorporated TDO (3EN1M-O_2 model), led to the predicted formation of catechols (54% docking iterations from **3a**, 34% from **3b**), but not of *cis*-dihydrodiols **5a** and **5b**. Further biotransformation (*P. putida* UV4) of catechols **13**, by a catechol dioxygenase-catalysed ring-opening process and other enzyme-catalysed reactions, gave a range of carboxylic acid metabolites. The formation of catechols, as arene metabolites, was also reported to inhibit the TDO activity, therefore reducing *cis*-dihydrodiol yields.[6k]

The predicted, and isolated, cyclohex-2-en-1-one tautomers, derived from phenols, *e.g.* **6a** and **6b**, were single enantiomers, having an (*S*) absolute configuration at C-5. The main attractive interactions, at the TDO active site, involved: (i) hydrogen bonding of the phenol OH group with the C=O group of Gln-215 and the imidazole ring of His-311, (ii) van der Waals interactions of the hydrophobic Me group of the phenol with the proximate alkyl (Ala-223, Val-309, Leu-321, Ile-324) and aryl (Phe-366) groups.

It was speculated that similar binding of phenols with His-311 and Gln-215, at the TDO active site, might also apply to aniline substrate interactions.[5f] An earlier *in silico* molecular binding model for diphenylamine **1** (R=NHPh, Scheme 1), at the active site of biphenyl dioxygenase (BPDO,) led to the prediction that *cis*-dihydroxylation would yield aniline *cis*-dihydrodiol intermediate **2** (R=NHPh).[6e] Although BPDO-catalysed *cis*-dihydroxylation of substituted aniline **1** (R=NHPh)[6d] did not result in the detection of *cis*-diol **2** (R=NHPh), it was postulated that formation of the major metabolite, phenol **4** (R= NHPh), had resulted from dehydration of this transient intermediate.[6f]

The qualitative nature of docking results recorded may not quantitatively reflect the experimental results (using *P. putida* UV4 whole cells), due to further metabolism by the co-induced enzymes. Apart from this caveat, the 3EN1M and 3EN1M-O_2 models, employed for TDO docking studies[5f] of phenol substrates **3**, were found to be useful predictors of the preferred regiochemistry and stereochemistry of *cis*-diol metabolites. These models have now been applied to *cis*-dihydroxylation of aniline substrates **9a-d** (Scheme 2). From analysis of the data collected, it was predicted that the NH-imines **11a-d** would be the preferred tautomers of the initially formed enamine *cis*-diols **10a-d**. It was also assumed that: (i) this type of imine would readily hydrolyse, during the biotransformation, to yield the corresponding cyclohex-2-en-1-one *cis*-diols **6a-d** and (ii) as observed,[5c] the formation of *cis*-triols **12**, *via* carbonyl reductase (CRED)-catalysed reduction of the ketone group in *cis*-diols **6**, would occur using *P. putida* UV4 whole cells. The 3EN1M and 3EN1M-O_2 model docking studies of anilines **9a-d** with TDO were also expected to provide evidence of preferred substrate orientations, leading to the formation of catechols **13a-d**, *via* alternative types of transient aniline *cis*-diol intermediates **14a-d**, resulting from *cis*-dihydroxylation at the 1,2- (*ipso*-*ortho*-) bond, as was proposed.[6c, 6e-i]

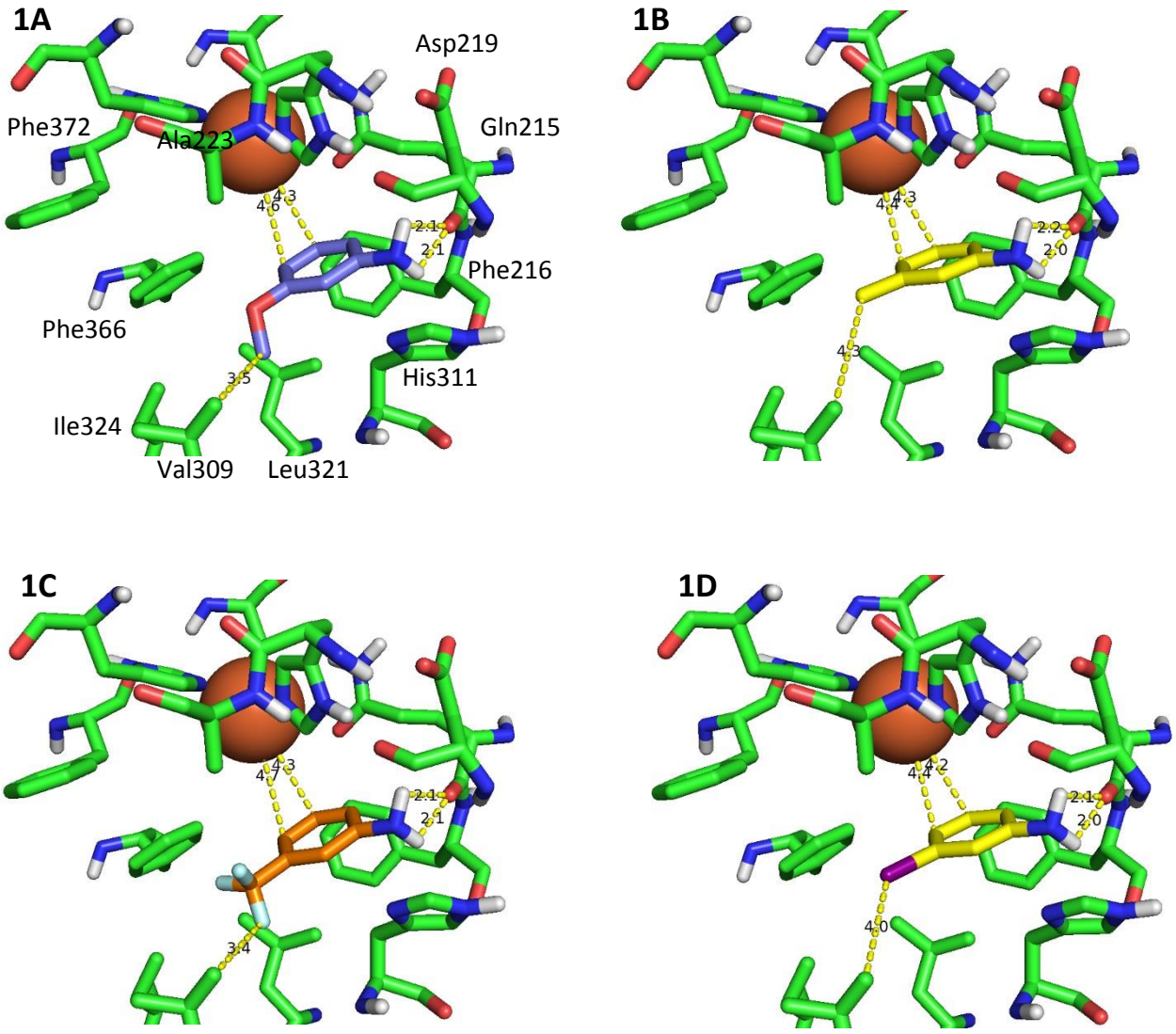
cis-Dihydroxylation, at the 4,5-bond and % formation of enamine *cis*-diols **10a-d**, was predicted (Table 1), from TDO docking orientations (using 3EN1M model), for *meta* substituted anilines **9a** (Fig. 1A, 47%), **9b** (Fig. 1B, 90%), **9c** (Fig. 1C, 80%) and **9d** (Fig. 1D, 100%). The substrate binding, according

Table 1. Predicted, using the 3EN1M model of TDO, initial metabolites **11a-d**, catechols **13a-c** and isolated *cis*-diol **6a-d** products of 3-substituted aniline substrates **9a-d**.

	Substrate	Figure	Predicted[a]	Product[b]	%[c]	ΔG[d]	K_D[e]
9a	**3-Methoxyaniline**	**1A**	**11a**	**6a**	47	**-5.08**	190.24
			13a		26	**-5.00**	216.49
9b	**3-Methylaniline**	**1B**	**11b**	**6b**	90	**-5.27**	137.66
			13b		4	**-5.14**	169.39
9c	**3-Trifluoromethylaniline**	**1C**	**11c**	**6c**	80	**-5.14**	171.53
			13c		20	**-5.07**	191.97
9d	**3-Iodoaniline**	**1D**	**11d**	**6d**	100	**-6.13**	31.88

[a] Predicted metabolite; [b] Detected product; [c] Orientation occurrence; [d] Binding energy (kJ/mol);[e] Dissociation constant (µM)

Figure 1. Molecular docking of *meta* substituted anilines **9a-d (**Figs **1A-1D)** at the active site of TDO



to the *in silico* docking, was facilitated by two major interactions: (i) van-der- Waals interaction of Ala-223, Val-309, Leu-321, Ile-324, Phe-366 and Phe-372 amino acid residues with the substituents (Me, OMe, CF_3, I) and (ii) H-bonding of the NH_2 group to Gln 215, and sometimes to His-311, in a similar manner to the docking of phenols.[5f]

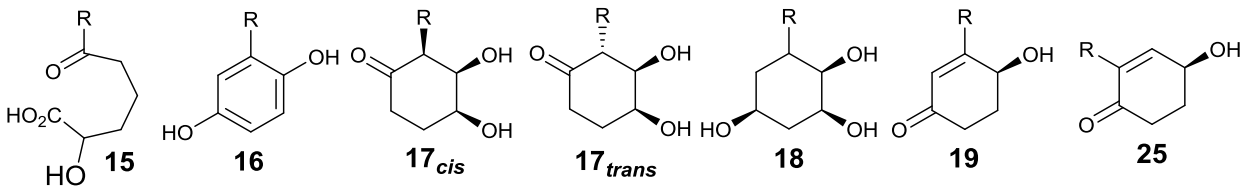
The docking experiments (3EN1M model) of anilines **9a-d** also led to the prediction that catechols **13a-c** might also be formed, *via* a minor pathway (4-26% docking orientations), by dihydroxylation at the 1,2-position, to yield intermediate *cis*-diols **14a-c**. Employing the 3EN1M-O_2 model of TDO, catechols **13a-d** were predicted to be the major metabolites (91-100% docking orientations), without evidence of orientations of anilines **9a-d**, leading to the corresponding *cis*-diols **10a-d** (Supporting Information Figs. S1-S4). These results (3EN1M-O_2 model) were similar to those found earlier for phenol substrates **3a** and **3b**, where catechols **13a** and **13b** were predicted to be the major metabolites (54 and 100% docking orientations) without evidence for the formation of *cis*-diols **5a** and **5b**.[5f]

Based on these predictive *in silico* docking (3EN1M model) studies of TDO (Table 1), experimental evidence was sought, for TDO-catalysed *cis*-dihydroxylation of *meta*-anilines **9a-d**, by LC-TOFMS analysis of the crude biotransformed culture medium. Aniline substrates **9a-d** were added, to *P. putida* UV4 cultures, under conditions similar to those reported for the corresponding *meta*-phenols **3a-d**.[5a-e] The cyclohex-2-en-1-one *cis*-diols **6a-d**, previously reported[5a-e] as phenol metabolites (Scheme 1**)**, were also detected as aniline metabolites (Scheme 2**)**, in accord with the predictions from *in silico* studies. The documented high cytotoxicity of anilines[6a,e] required a ten-fold reduction of the substrate concentration (0.05 mg/mL), for total conversion. Phenols **3a-d** and corresponding anilines **9a-d**, applied in the same low concentrations, produced comparable yields of cyclohexenone *cis*-diols **6a-d**, which were identified by comparison (LC/TOFMS and GC-MS) with authentic samples. A sample of *cis*-diol **6a** (*ca*. 6 mg) was also isolated by PLC, from the partial biotransformation using a higher concentration of aniline **9a**; its structure, absolute configuration (4*S*,5*S*) and enantiopurity (>98% *ee*) was found to be identical with the metabolite derived from phenol **3a**. From this result, combined with the *in silico* docking studies (Figs. 1A-D), it was predicted that the (*S*)-absolute configuration at the C-5 position and *ee* value (>98%) of *cis*-diol metabolites **6b-d**, derived from the corresponding aniline substrates **9b-d**, would be identical to those obtained from phenols **3b-d**.

The first objective of the study was to provide experimental evidence for TDO-catalysed *cis*-diol formation from aniline substrates, but the presence of other metabolites extended our interest into exploring the complete metabolic profile of anilines with the *P. putida* biocatalyst. A CRED enzyme, present in *P putida* UV4, was previously found to catalyse the reduction of the ketone group of metabolite **3c**, to yield (1*R*,2*S*,4*R*)-6-(trifluoromethyl)cyclohex-5-ene-1,2,4-triol **12c** as a major bioproduct.[5c] A similar result was obtained with aniline substrate **9c** when metabolites *cis*-diol **6c** and triol **12c** were identified by LC-TOFMS analysis.

Catechols were identified as aniline metabolites[6a-i] and molecular docking (3EN1M and 3EN1M-O_2 models) experiments of TDO also suggested their formation from anilines **9a-d** (Table 1). LC-TOFMS analysis did not show direct evidence of catechol metabolites **13a-d**, but indirect evidence, for the formation of catechol metabolite **9a**, was observed by the formation of a carboxylic acid metabolite, whose molecular weight was consistent with structure **15a**, formed by catechol dioxygenase-catalysed ring opening and reductase-catalysed reduction (**Fig.2**). Similar ring-opened metabolites **15** (R = Me, CF_3) were previously reported from the corresponding phenols (**3b** and **3c**) and catechols (**13b** and **13c**).[5c] GC-MS analysis of trimethylsilylated samples, prepared from freeze-dried aliquots collected during TDO-catalysed dihydroxylation of anilines **9a** and **9c**, showed the presence of disilylated *cis*-diol **6a** and **6c** and derived hydroquinones **16a** and **16c** respectively, but no catechols were detected. The difficulty encountered in the detection of catechol metabolites **13a-d** was probably due to: (i) the activity of a catechol dioxygenase enzyme present in *P. putida* UV4 and (ii) the low yields of all metabolites resulting from the cytotoxicity of aniline substrates.

Biotransformations of anilines **9a-d**, with the recombinant strain, *E. coli* pCL-4t (expressing TDO), and LC-TOFMS analysis of the biotransformed aqueous material, again showed the presence of



R = OMe (a), Me (b), CF_3 (c), I (d), H (e)

Figure 2. Structures of metabolites **15-18** and **25**.

cyclohexenone *cis*-diol metabolites **6a-d**. The reduced tolerance of *E. coli* cells, to the toxic aniline substrates, gave lower yields, compared with those found using *P. putida* UV4 cells. However, the *E. coli* pCL-4t biotransformation studies did provide evidence that TDO, rather than other types of dioxygenase, was responsible for the formation of *cis*-diols **6a-d**.

The biotransformation and molecular docking results, recorded for aniline substrates **9a-d**, were consistent with a metabolic pathway *via* TDO-catalysed formation of enamine *cis*-dihydrodiols **10a-d**, tautomerisation to the preferred NH-imine *cis*-diols **11a-d**, and rapid hydrolysis to yield cyclohex-2-en-1-one *cis*-diols **6a-d** (Scheme 2**)**. Thus, the family of cyclohex-2-en-1-one *cis*-diol metabolites is formed, from both substituted phenols and anilines, by TDO-catalysed *cis*-dihydroxylation. The molecular docking results can also be used to rationalize the reported formation of catechol and hydroquinone metabolites of aniline substrates,[6a-j] *via* ring hydroxylating dioxygenase catalysis.

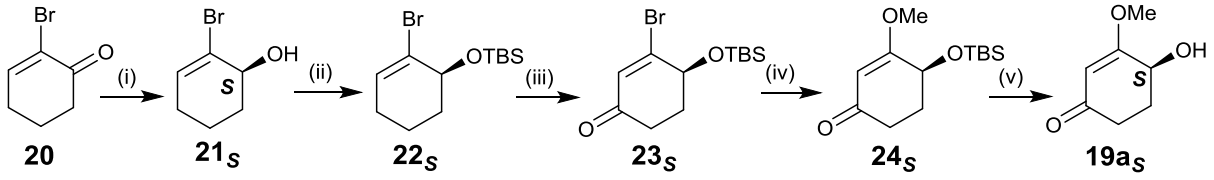
(ii) Biotransformations of phenol substrates, to yield 4-hydroxycyclohex-2-en-1-ones

Earlier larger scale biotransformations of *meta*- and *ortho*-phenols **3** and **4**, showed a wide range of metabolite types, including cyclohexenone *cis*-diols **6** and **8**, cyclohexene *cis*-triols **12**, catechols **13**, α-hydroxycarboxylic acids **15**, hydroquinones **16**, cyclohexanone *cis*-diol isomers $\mathbf{17}_{cis}$ and $\mathbf{17}_{trans}$ and 1,2,4-trihydroxycyclohexanes **18**[5a-e] **(Scheme 1 and Fig. 2).** Several minor metabolites of methoxyphenols **3a** and **4a**, however, remained unidentified;[5e] their structures, absolute configurations and metabolic pathways for their formation are presented in this section.

Recrystallization of the crude mixture of metabolites, obtained from an earlier biotransformation of 3-methoxyphenol **3a** (96 g), with glucose as carbon source, yielded *cis*-diol **6a** as the major component (38% isolated yield, Scheme 1).[5e] The mother liquors from this recrystallization contained a mixture of unidentified minor metabolites, which were examined further during this study. Column chromatography of the mixture yielded a new minor metabolite (1% isolated yield), which was structurally identified as 4-hydroxy-3-methoxycyclohex-2-en-1-one **19a** ($[\alpha]_D$ -31.9).

A chemoenzymatic synthesis of metabolite **19a**, starting from 2-bromo-2-cyclohexen-1-one **20** established (4*S*) as its absolute configuration (Scheme 3). Step (i) employed a CRED-catalysed reduction of the ketone group of 2-bromo-2-cyclohexen-1-one **20**, to give the enantiopure synthetic precursor cyclohexenol $\mathbf{21}_S$ ($[\alpha]_D$ -80.3, 88%).[5h] Further chemical steps involved, hydroxyl group protection (ii, $\mathbf{21}_S \longrightarrow \mathbf{22}_S$, 94%), allylic oxidation (iii, $\mathbf{22}_S \rightarrow \mathbf{23}_S$, 29%), nucleophilic substitution (iv, $\mathbf{23}_S \longrightarrow \mathbf{24}_S$, 69%) and deprotection (v, $\mathbf{24}_S \longrightarrow \mathbf{19a}_S$, 62%, Scheme 3). This synthetic sample of compound $\mathbf{19a}_S$ (>98% *ee*) had a higher optical rotation ($[\alpha]_D$ -48.8), compared with the corresponding metabolite ($\mathbf{19a}_S$) derived from phenol **3a**. The lower enantiopurity (65% *ee*) of the minor metabolite, 4-hydroxy-3-methoxycyclohex-2-en-1-one $\mathbf{19a}_S$, compared to the major metabolite, (4*S*,5*S*)-3-methoxycyclohex-2-ene-1-one **6a** (>98% *ee*), was of mechanistic relevance, in the context of biosynthetic pathways from 3-methoxyphenol **3a** (Scheme 4), that will be discussed in Section (iii).

A previous biotransformation (glucose as carbon source) of 2-methoxyphenol **4a** (96 g), followed by column chromatography, resulted in a separable mixture of isomeric cyclohexanone *cis*-diols, (2*S*,3*S*,4*S*)-$\mathbf{17a}_{cis}$ (13% isolated yield) and (2*R*,3*S*,4*S*) $\mathbf{17a}_{trans}$ (1% isolated yield).[5e] Metabolites $\mathbf{17a}_{cis}$ and $\mathbf{17a}_{trans}$ were formed *via* an ene reductase (ERED)-catalysed reduction of the initial bioproduct, cyclohex-2-en-1-one *cis* diol **8a** (Scheme 1). Using LC-TOFMS and GC-MS analyses, the relative ratios of metabolites from guaiacol **4a** were found to vary widely, during time course studies of the biotransformations, depending on the choice of carbon



Reagents: (i) CRED / [2H]; (ii) TBSOTf, NEt_3, CH_2Cl_2: (iii) $PhI(OAc)_2$, K_2CO_3, t-BuO_2H; (iv) K_2CO_3, MeOH; (v) TBAF, THF.

Scheme 3. Chemoenzymatic synthesis of 4-hydroxy-3-methoxycyclohex-2-en-1-one $\mathbf{19a}_S$ from 2-bromo-2-cyclohexen-1-one **20**.

source (glucose or pyruvate) and TDO source (*P. putida* or *E. coli*). Other metabolites from phenol **4a** were identified as cyclohex-2-en-1-one *cis* diol **8a**, hydroquinone **16a**, catechol **13a** and its α-hydroxycarboxylic acid derivative **15a.**

Column chromatography fractions, from the earlier study,[5e] that appeared to be an inseparable mixture of two unidentified isomeric metabolites of phenol **4a** were retained for further examination. During the current study, this mixture, was finally separated by careful multiple elution PLC. The minor isomer (2% isolated yield) was indistinguishable from 4-hydroxy-3-methoxycyclohex-2-en-1-one **19a$_S$** derived from 3-methoxyphenol **3a**. The structure and absolute configuration of the major isomer (18% isolated yield, $[\alpha]_D$ -29), was identified as (4*S*)-4-hydroxy-2-methoxycyclohex-2-en-1-one **25a$_S$**. This metabolite was also isolated as a dehydration product of (2*S*,3*S*,4*S*)-3,4-dihydroxy-2-methoxycyclohexanone **17a$_{cis}$** (Scheme 4).

A repeat biotransformation of guaiacol **4a** again resulted in the formation of chiral metabolites **17a$_{cis}$**, **25a$_S$** and **19a$_S$**, but in a different ratio based on isolated yields, *i.e.* 11%, 3%, <1% respectively. This prompted a time course biotransformation study of methoxyphenol substrates **3a** and **4a**, which showed increases in the relative yields of 4-hydroxy-3-methoxycyclohex-2-en-1-ones **19a$_S$** and **25a$_S$** respectively, with glucose, rather than pyruvate, as a carbon source and during the later stages (>8 h) of the biotransformations.

The unexpected discovery of 4-hydroxymethoxycyclohex-2-en-1-ones **19a$_S$** and **25a$_S$**, metabolites of phenol substrates **3a** and **4a**, allied to the earlier isolation of compound **19e$_R$**, as a minor metabolite of 3-iodophenol **3d**,[5a] raised the possibility that compounds **19a$_S$**, **25a$_S$** and.**19e$_R$** could be the first members of a new family of phenol metabolites. To investigate the possible metabolic pathways, leading to the formation of 4-hydroxycyclohex-2-en-1-ones **19a$_S$**, **19e$_R$** and **25a$_S$**, repeat biotransformations of 3-iodophenol **3d** and *ortho*-cresol **4b** were conducted (Scheme 4).

Iodocyclohex-2-en-1-one *cis*-diol **6d**, a major metabolite of 3-iodophenol **3d**,[5a,c] was isolated in variable yields (30-70%), along with other metabolites including 4-hydroxycyclohex-2-en-1-one **19e$_R$**. LC-TOFMS analysis of the biotransformed aqueous culture medium, detected the presence of cyclohexanone *cis*-diol **26e** and GC-MS analysis of the EtOAc concentrate, after trimethylsilylation with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), showed that iodocyclohexene *cis*-triol **12d**, cyclohexane *cis*-triol **18e**, iodocatechol **13d**, iodohydroquinone **16d** and cyclohexene *cis*-triol **12** (R=H) were also present as minor metabolites (Scheme 2, 4 and Fig 2).

A preliminary biotransformation of *ortho*-cresol **4b**, resulted in the isolation of (4*S*,5*R*,6*S*)-4,5-dihydroxy-6-methylcyclohex-2-en-1-one **8b**, as the only identified metabolite (1% yield).[5a] Repeated metabolism studies of phenol **4b**, revealed that in addition to *cis*-diol **8b**, four other minor metabolites were present. Catechol **13b** and hydroquinone **16b** were identified by trimethylsilylation of a small portion of the crude freeze-dried extract, followed by GC-MS analysis of the products and comparison with authentic samples.

Time course LC-TOFMS analysis, of the crude culture medium from a biotransformation of *ortho*-cresol **4b**, indicated that, in addition to **8b**, a very minor bioproduct was formed, which rapidly metabolized further. This early eluting bioproduct, was tentatively identified as cyclohexanone *cis*-diol **17b$_{cis}$** (Scheme 4). Its identity was confirmed by catalytic hydrogenation (Pd/C, MeOH) of metabolite **8b**, to yield an identical sample of (2*S*,3*R*,4*S*)-3,4-dihydroxy-2-methylcyclohexanone **17b$_{cis}$**. Work up of the biotransformed material[5a] and separation of the crude mixture, by column chromatography followed by PLC of early eluting fractions, gave a new metabolite, which was identified as 4-hydroxy-2-methylcyclohex-2-en-1-one **25b$_S$** ($[\alpha]_D$ - 49.0). The opposite enantiomer, **25b$_R$**, ($[\alpha]_D$ + 46.7), had been synthesised earlier by an alternative route, involving manganese acetate-mediated acetoxylation and lipase-catalysed ester hydrolysis.[7]

(iii) Biosynthetic pathways for the formation of 4-hydroxycyclohex-2-en-1-one - metabolites from phenols

The metabolic sequences, to account for the formation of the 4-hydroxycyclohex-2-en-1-one metabolites **19e$_R$**, **19a$_S$**, **25a$_S$** and **25b$_S$**, as minor products from the corresponding phenol substrates (**3d**, **3a**, **4a** and **4b**), *via* the initial cyclohexenone *cis*-diol metabolites **6d**, **6e**, **6a**, **8a** and **8b**, are presented in Scheme 4.

It is proposed that, during the *P. putida* UV4 biotransformation of 3-iodophenol **3d**, an ERED-catalysed ene reduction of cyclohexenone *cis*-diol **6d**, followed by a dehydrohalogenation of the resulting cyclohexanone *cis*-diol could occur, to give the transient parent cyclohex-2-en-1-one *cis*-diol **6e** (Scheme 4). Intermediate **6e** was not detected, possibly due to its further rapid ERED-catalysed ene reduction to form the detected transient metabolite **26e**. CRED-catalysed reduction of metabolite **26e** yielded the *cis*-triol metabolite **18e** (Fig. 2, R = H), while its facile

dehydration also gave 4-cyclohex-2-ene-1-one **19e$_R$**. A precedent for this type of reductive dehalogenation mechanism, (**6d** ⟶⟶ **6e**) by the ERED-catalysed reduction-spontaneous *β*-elimination of *β*-halo-*α,β*-unsaturated carboxylic esters, has been reported.[8] Other examples of ERED-catalysed ene reductions, of *α,β*-unsaturated ketones, *e.g.* metabolites **8a**→**17a$_{cis}$** and **8b**→**17b$_{cis}$**, have been found during biotransformations[5e] (Scheme 4).

Further evidence of the metabolic sequence (**3d**⟶**6d**⟶⟶**6e**⟶**26e**⟶**19e$_R$**), involving both TDO and ERED enzymes, was found when cyclohex-2-en-1-one *cis*-diol **6e** ([*α*]$_D$ - 217), obtained by hydrogenolysis of metabolite **6d**, was added as substrate. 4-Hydroxycyclohex-2-en-1-one **19e$_R$** and triol **18e** were the only identified metabolites. Enantiopure 4-hydroxycyclohex-2-en-1-one **19e$_R$**, ([*α*]$_D$ +110) synthesised by an alternative chemoenzymatic route using lipase enzymes, has been utilized as a chiral precursor in synthesis.[9a,b]

Biosynthetic sequences, involving TDO-catalysed *cis*-dihydroxylation of phenols **4a** and **4b**, to yield enantiopure cyclohex-2-en-1-one *cis*-diols followed by an ERED-catalysed reduction / *β*-elimination mechanism, are shown in Scheme 4. The metabolic pathway proposed for the formation of 4-hydroxycyclohex-2-en-1-one **25a$_S$** from phenol **4a**, (**4a**⟶**8a**⟶**17a$_{cis}$**⟶**25a$_S$**), was supported by results obtained using (2*S*,3*S*,4*S*)-cyclohexanone *cis*-diol **17a** as substrate; compound **25a$_S$** was the only bioproduct formed. It was presumed that the biotransformation of phenol **4b**, to yield 4-hydroxycyclohex-2-en-1-one **25b$_S$**, would also occur *via* a similar metabolic pathway (**4b**⟶**8b**⟶**17b$_{cis}$**⟶**25b$_S$**).

While the formation of three 4-hydroxycyclohex-2-en-1-ones (**19e$_R$**, **25a$_S$** and **25b$_S$**), from the corresponding phenol substrates (**3d**, **4a** and **4b**), resulted from a common biosynthetic sequence, this pathway would not result in the formation of metabolite **19a$_S$** from phenol **3a**. Furthermore, the higher enantiopurity (>98% *ee*), of bioproducts **19e$_R$**, **25a$_S$** and **25b$_S$** from phenols **3d**, **4a** and **4b**, contrasted with the lower *ee* value of metabolite **19a$_S$** (*ca*. 65% *ee*, from phenol **3a**) found earlier. This indicates the probability of a different mechanism being involved in the formation of 4-hydroxycyclohex-2-en-1-one **19a$_S$** from phenols **3a** and **4a**.

With cyclohexenone *cis*-diol **6a** as substrate, 4-hydroxycyclohexenone **19a$_S$** was identified as the main metabolite with hydroquinone **16a** as a minor product resulting from the dehydration. Since hydroquinone **16a** also being formed by the dehydration of cyclohexenone *cis*-diol **8a**, its possible role as an intermediate during formation of 4-hydroxycyclohex-2-en-1-one **19a$_S$** was examined. The biotransformation pathways of phenols **3a** and **4a**, with hydroquinone **16a** as an intermediate, were postulated to proceed in five steps (**3a**⟶**6a**⟶**16a**⟶**27a**⟶**28a**⟶**19a$_S$** and **4a**⟶**8a**⟶**16a**⟶**27a**⟶**28a**⟶**19a$_S$**) as shown in Scheme 4. Further confirmation of these metabolic sequences was obtained by the biotransformation of hydroquinone **16a** as substrate to yield metabolite 4-hydroxycyclohex-2-en-1-one **19a$_S$**.

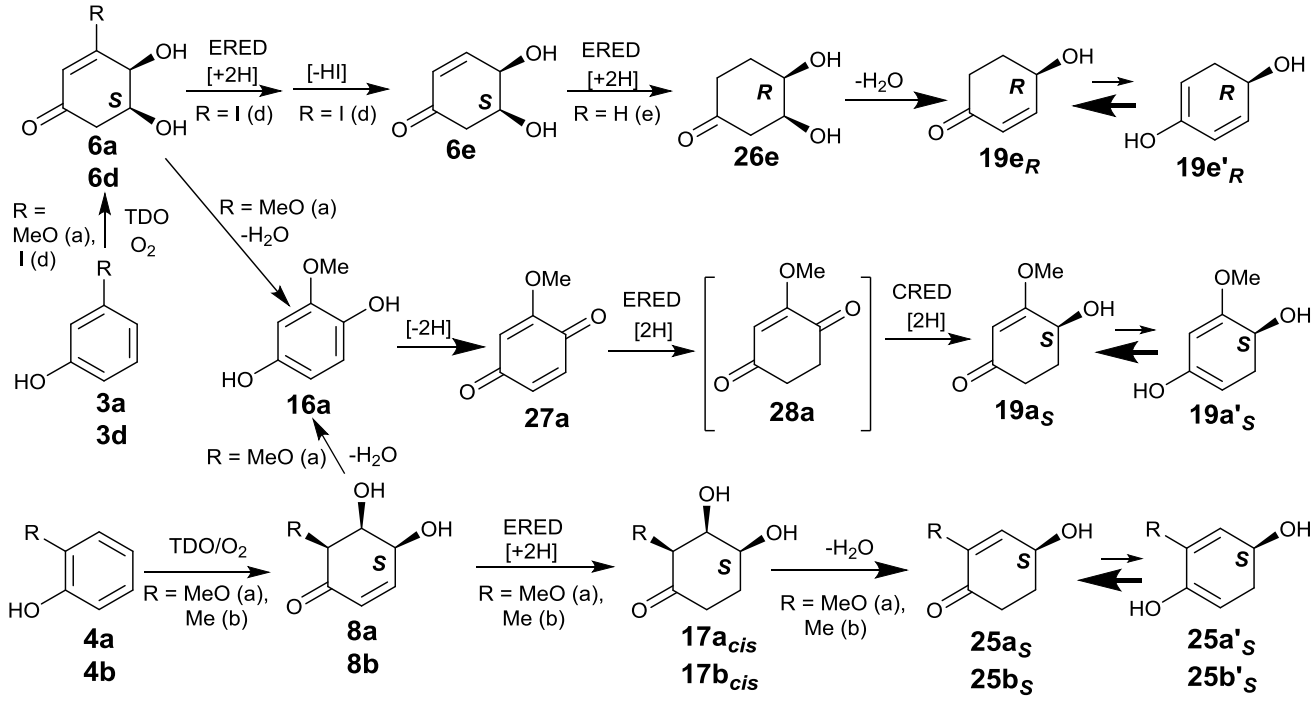
It is postulated that the oxidation of hydroquinone **16a**, to benzoquinone intermediate **27a**, could have resulted from either a non-enzymatic autoxidation or peroxidase activity in *P. putida* UV4 cells, followed by an ERED-catalysed reduction of benzoquinone **27a** (Scheme 4). The transient intermediate, cyclohex-2-ene-1,4-dione **28a**, could either tautomerize back to hydroquinone **16a** or undergo an asymmetric CRED-catalysed ketone reduction. It could account for metabolite **19a$_S$** having a lower enantiopurity (65% *ee*) compared with *cis*-diol precursors **6a** and **8a** (>98% *ee*).

Fungal metabolism of benzoquinone **29**, with cultures of *Phanerochaete chryosporium*,[10] to form 4-hydroxycyclohex-2-enone **30** (Fig.3) provides a precedent for the metabolic sequence (**27a**⟶**28a**⟶**19a$_S$**) Dehydration of cyclohexenone *cis*-diols **6a** and **8a**, to give hydroquinone **16a**, and of cyclohexanone *cis*-diols **17a**, **17b** and **26e**, to form 4 - hydroxycyclohex-2-en-1-ones **25a$_S$**, **25b$_S$** and **19e$_R$** respectively, could occur during biotransformations, *via* chemocatalysis or a dehydratase-catalysed process.

(iv) Biotransformations of monocylic arenes, to yield arene hydrates

The hydration of conjugated and non-conjugated alkene bonds, catalysed by hydratase or hydrolyase enzymes, *e.g.* aconitase, fumarase and crotonase, is a common step in primary metabolism.[11a] There are very few reported examples of enzymatic hydrations of arenes, to form the corresponding arene hydrates. The formation of arene hydrate metabolite **31** from acetophenone substrate **1** (R = COMe) using *P. putida* UV4 cultures, is among the few reports of arene to arene hydrate biotransformations (Scheme 1, Fig. 3).[11b]

Metabolite **31**, a highly unstable compound, with a propensity to rapidly dehydrate back to substrate **1** (R = COMe), was only identified as an iron tricarbonyl complex.[11b] To study the stability of monocyclic arene hydrates, racemic samples of compounds **32** (R = Me, Et, ^{i}Pr, ^{t}Bu), and **33** (R = CO_2Me and Ph), were synthesised from 3-substituted



Scheme 4. Metabolic pathways for the formation of 4-hydroxycyclohex-2-en-1-ones **19e*R***, **19a*S***, **25a*S*** and **25b*S***, from phenols **3d**, **3a**, **4a** and **4b** respectively.

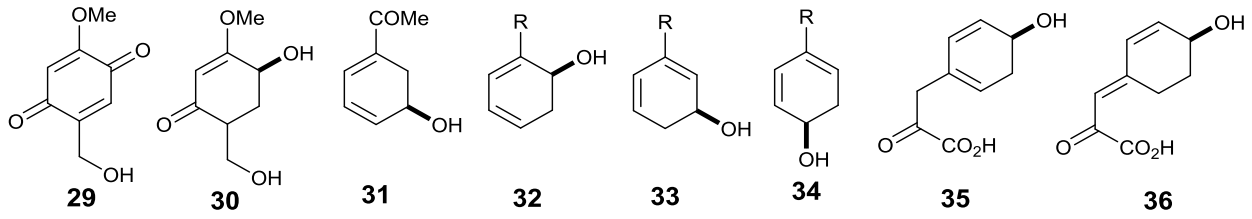


Figure 3. Structures of compounds **29-36**.

1,4-cyclohexadienes,[11c] and enantiopure arene hydrates **33** (R = F, Cl, Br. CF_3) and **34** (R = Br) from the corresponding *cis*-dihydrodiol metabolites **2**.[11d] Kinetic studies of the acid-catalysed dehydration of these arene hydrates showed that they aromatized much faster ($3.7x10^2$ - $6.9x\ 10^4$ fold) than *cis*-dihydrodiols **2**.[11c]

The monocyclic arene hydrate **35**, an unstable intermediate, formed during the biosynthesis of the antibiotic bacilysin, was obtained by the enzymatic decarboxylation of prephenate, using an *E. coli* recombinant strain, expressing phenate decarboxylase.[12a-c] Arene hydrate **35** was found to undergo a slow non-enzymatic, or a rapid enzyme-catalysed isomerization, to yield the more stable vinylogous 4-hydroxycyclohexenone metabolite **36**. Intermediate **35** appears to be among the very few, isolated and fully characterized, monocyclic arene hydrate metabolites.

Phenol metabolites, 4-hydroxycyclohex-2-en-1-one **19e*R***, **19a*S***, **25a*S*** and **25b*S***, identified during the study could, in principle, equilibrate with the corresponding phenol hydrate tautomers **19e'*R***, **19a'*S***, **25a'*S*** and **25b'*S***; in practice only the keto tautomers were observed by NMR spectroscopy. Similarly, the methoxycyclohexenone *cis*-diol **6a**, metabolite of 3-methoxyphenol **3a**, showed no evidence of enol tautomer **5a**, from proton NMR analysis (Scheme 1). Trimethylsilylation of *cis*-diol **6a** with MSTFA, and GC-MS analysis of the product, showed a major peak (95%) with ($[M]^+$ *m/z* 302), indicating formation of the diTMS derivative.[5e] The molecular ion ($[M]^+$ *m/z* 374), corresponding to the minor peak (5%), was from a triTMS derivative and provided indirect evidence of

the elusive enol tautomer **5a**. Similar treatment of 4-hydroxycyclohex-2-en-1-ones **19e**$_R$ and **19a**$_S$ with MSTFA, and GC-MS analyses of the products showed the formation of monoTMS derivatives **19e'**$_R$ and **19a'**$_S$ (major peaks). The minor peaks were attributed to the diTMS derivatives of the undetected phenol hydrate tautomers **19e'**$_R$ and **19a'**$_S$.

The lack of evidence, for monocyclic arene hydrates of similar structure to metabolite **31**,[11b] during the biotransformations of substituted benzene substrates by *P. putida* UV4 or other microbial systems could be due to the absence of suitable hydrolase / hydratase enzymes or rapid dehydration and reformation of the arene substrates.[11c] Under similar biotransformation conditions, the TDO-catalysed *cis*-dihydroxylation of arene hydrate **33** (R = CF_3) yielded the opposite enantiomer of cyclohexene triol **12c**,[11d] and thus revealed an alternative metabolic pathway for monocyclic arene hydrates. The results presented herein demonstrate that the more stable keto tautomers of arene hydrates can be obtained from phenols using *P. putida* UV4.

Conclusion

Molecular docking results, of four aniline substrates with TDO, led to the prediction that in common with phenols, *cis*-dihydroxylation of anilines could occur, to yield *cis*-diols and catechols. The premise was confirmed by the detection and isolation, of identical cyclohex-2-en-1-one *cis*-diol metabolites, from the corresponding *meta*-phenols and *meta*-anilines. The initial formation of enamines and the NH-imine tautomers, followed by their rapid hydrolysis, could account for the formation of cyclohex-2-en-1-one *cis*-diols in low yields from the anilines. Although catechols had been found earlier as aniline metabolites, no direct evidence was found in the study, possibly due to further metabolism by catechol dioxygenase.

Biotransformation of cyclohex-2-en-1-one *cis*-diols, resulted in the formation of a new range of minor metabolites, which were identified as 4-hydroxycyclohex-2-en-1-ones. Their structures and absolute configurations were determined by chemoenzymatic synthesis and stereochemical correlation and have considerable synthetic potential.

Although single step hydratase or hydrolase activity has been reported for alkenes, we are unaware of similar activity with arene substrates. Multistep metabolic pathways, involving cyclohexenone *cis*-diol, cyclohexanone *cis*-diol and hydroquinone intermediates, are now proposed to explain the formation of 4-hydroxycyclohex-2-en-1-ones as a new type of phenol metabolite. Although direct evidence for the presence of enol tautomers (arene hydrates) of 4-hydroxycyclohexenones was not found, indirect evidence was obtained following trimethylsilylation and GC-MS analyses.

Experimental Section

Experimental Details

NMR spectra were recorded on Bruker Avance-400, DPX-300 and DRX-500 instruments. Chemical shifts (δ) are reported in ppm relative to $SiMe_4$, and coupling constants (J) are given in Hz. IR spectra were recorded on a Perkin-Elmer 983G spectrometer. Optical rotation ($[\alpha]_D$) measurements were carried out on a Perkin-Elmer 214 polarimeter. LC-TOFMS analyses were conducted using an Agilent 1100 series HPLC coupled to an Agilent 6510 Q-TOF (Agilent Technologies, USA) and a reverse phase column (Agilent Eclipse Plus C18, 5 mm, 150 x 2.1 mm) under reported conditions.[5e] GC-MS analysis of metabolites was carried after silylation using MSTFA and an Agilent Technologies 6890N gas chromatograph linked to a 5973 mass selective detector and Agilent Technologies HP Ultra 2 column as reported.[5e] For TLC analysis, Merck Kieselgel $60F_{254}$ analytical plates were used and PLC separation of metabolites was carried out using glass plates (20 cm x 20 cm) coated with Merck Keiselgel $PF_{254/366}$ silica gel. Column chromatography was performed on Merck Keiselgel type 60 (250-400 mesh).

Phenols **3a-3d**, **4a**, **4b**, anilines **9a-d**, catechols **13a**, **13b**, hydroquinones **15a**, **15b** and methoxybenzoquinone **27a** were purchased from Sigma-Aldrich Co. Authentic samples of cyclohex-2-en-1-one *cis*-diols **6a-d** and cyclohexene *cis*-triol **12c** were available from earlier studies.[5a-e] Commercially obtained anilines **9a-d** showed no detectable traces of phenols **4a-d** on GC-MS analysis, prior to their use as substrates. Biotransformations were carried out using *P. putida* UV4 cells, unless mentioned otherwise.

Molecular modelling

Substrate docking studies were performed according to an earlier procedure.[5f] The required *in silico* models of substrates were created in .pdb-format with UCSF Chimera 1.10.2 (https://www.cgl.ucsf.edu/chimera/). *In silico* dockings were performed with AutoDock suite 4.2 (autodock4, autogrid4). The Graphical User Interface (GUI), including python scripts for ligand and receptor preparation, was part of AutoDock Tools 1.5.6. AutoDock suite and AutoDock tools (ADT) are provided by the Scripps Research Institute (http://autodock.scripps.edu/).[13] The TDO crystal structure was accessed from the Protein Data Bank (PDB code 3en1, resolution of 3.2 Å). The raw 3en1 crystal structure .pdb-file of TDO includes a docked toluene structure in the active site, which was removed with UCSF chimera 1.10.2 prior to docking. The resulting model was called 3EN1M. Ligand and receptor were then prepared in accordance with the ADT tutorial

(http://autodock.scripps.edu/faqs-help/tutorial/using-autodock-4-with autodocktools/2012_ADTtut.pdf) utilising the 'prepare_receptor4.py' python script included in ADT, missing atoms were repaired, hydrogens and Gasteiger charges added, and non-polar hydrogens merged. The resulting .pdbqt-file of the crystal structure was used in all docking calculations. .pdb-files of substrates were automatically converted to the required .pdbqt-format by ADT. The docking grid was adjusted to include all amino acids within 5 Å of toluene in the crystal structure: Gln215, Phe216, Asp219, Met220, His222, Ala223, His228, Val309, His311, Leu321, Ile324, Phe366, Phe376.

PyMol was used to incorporate dioxygen into the 3EN1M model, by superimposing the iron complex (Fe, His222, His228, and Asp376) of TDO (pdb code: 3en1) with that of NDO (pdb code: 1o7m), and copying the dioxygen positions to 3EN1M. The resulting model was called 3EN1M-O_2.

Docking resolution = 0.247 Å; x_{size} = 40; x_{offset} = 12.833; y_{size} = 60; y_{offset} = -4.472; z_{size} = 72; z_{offset} = 3.917. The grid parameter file (.gpf) was used to create the grid map files (.glg) using autogrid4. The search protocol for docking used the internal default docking parameters of AutoDock 4.2, starting the ligand at a random location. The docking was set to be performed as 100 runs of the Lamarckian genetic algorithm with a population size of 150 each, terminating after 2 500 000 energy evaluations or 27 000 generations (whichever occurs first; standard settings). The docking results were analysed with ADT, to obtain docking coordinates, calculated binding energy (in kJ) and calculated dissociation constant K_D (in μM). All docking studies were performed as rigid docking, keeping all positions, orientations and protonation states of all amino acid atoms locked in place. The 100 docking orientations were automatically analysed by ADT and divided into orientationally and energetically similar groups. The orientation with the highest binding energy of each group was saved as representative for each group. The conformation of toluene, in the crystal structure, was used as a reference, to establish and confirm the viability of the docking procedure and parameters, as a similar conformation was obtained from docking with ADT.

3D visualization

The amino acids of the TDO active site were visualized with PyMol 1.7.4.5, from the Protein Data Bank (PDB) 3EN1 file coordinates. The docking results were imported into PyMol, from the respective .pdb files created with AutoDock4 and AutoDock Tools. Measurements between atoms were calculated with PyMol's incorporated measurement tool.

Biotransformation of anilines 9a-d

Initial small scale biotransformations of anilines **9a-d** and LC-TOFMS analyses of metabolites were conducted, under conditions similar to those reported for phenol substrates.[5a-e] However, due to the increased toxicity of anilines **9a-d**, compared with phenol substrates **3a-d**, a significant proportion of residual aniline substrates were consistently present along with cyclohexenone *cis*-diol metabolites **6a-d**. To achieve complete conversions, lower aniline concentrations were used (*ca*. 0.05 mg/ml). Cyclohexenone *cis*-diol metabolites **6a-d** and *cis*-triol **12c** were readily identified as aniline metabolites, by comparison of LC-TOFMS data with the authentic samples. As cyclohexenone *cis*-diols **6a-d** and *cis*-triol **12c** had been fully characterised [5a-e], only the LC-TOFMS retention times (min.) and accurate mass values were recorded for aniline substrates **9a-d**.

3-Methoxycyclohex-2-en-1-one *cis*-diol 6a.[5d] 3.43 min., HRMS: $[M+H]^+$ 159.0646, calcd. for $C_7H_{11}O_4$ 159.0652.

3-Methylcyclohex-2-en-1-one *cis*-diol 6b.[5a] 4.85 min., HRMS: $[M+H]^+$ 143.0698, calcd. for $C_7H_{11}O_3$ 143.0703.

3-Trifluoromethylcyclohex-2-en-1-one *cis*-diol 6c.[5c] 10.93 min., HRMS: $[M+H]^+$ 197.0417, calcd. for $C_7H_8O_3F_3$ 197.0420.

3-Iodocyclohex-2-en-1-one *cis*-diol 6d.[5a] 10.19 min., HRMS: $[M+H]^+$ 254.9502, calcd. for $C_6H_8O_3I$ 254.9513.

6-Trifluoromethylcyclohex-5-ene *cis*-triol 12c.[5c] 9.65 min., HRMS: $[M+NH_4]^+$ 216.0831, calcd. for $C_7H_{13}NO_3F_3$ 216.0847.

Cyclohex-2-en-1-one *cis*-diol metabolite **6a** (*ca*. 6 mg) was isolated from a biotransformation of aniline **9a** (1.25 g in 4 L culture medium). Purification, by column chromatograph (hexane → EtOAc), of the crude product obtained after usual work up followed by PLC (50% EtOAc in hexane), of selected combined fractions, gave metabolite **6a**. The metabolite was found to have identical spectroscopic (NMR) and chiroptical ($[\alpha]_D$) properties to an authentic sample derived from phenol **3a**. No catechol metabolites [6a-e] were detected (GC-MS analysis) in the crude bio-extracts of aniline substrates **9a-d**.

Biotransformation of phenols 3a, 3d, 4a and 4b and synthesis of (-)-(*S*)-4-hydroxy-3-methoxycyclohex-2-en-1-one 19a*S*

Large scale biotransformations of phenols **3a** and **4a**, using whole cell cultures of *P. putida* UV4 with glucose as a carbon source, were reported.[5e] Pooled column fractions, from that investigation containing unidentified metabolites, were re-examined during the current study. Time course study of small scale biotransformations of phenols **3a**, **3d**, **4a** and **4b** and anilines **9a-d**, were conducted under similar conditions, using glucose or pyruvate as carbon sources.[5a-e] Chiral metabolites, 4-hydroxycyclohex-2-en-1-ones **19a**, **19e, 25a** and **25b**, cyclohexenone *cis*-diols **6a** and **6d**, cyclohexanone *cis*-diols **17a**, **17b** and **26e**, and *cis*-triol **12c** were detected directly in the crude aqueous culture medium by LC-TOFMS analyses, prior to their isolation. Catechols **13a-d**, hydroquinones **16a-d** were identified by GC-MS analyses of the crude bio-extracts, after EtOAc extraction and trimethylsilylation (MSTFA) of the dried concentrates.

(a) 4-Hydroxy-3-methoxycyclohex-2-en-1-one 19a*S*a new metabolite of 3-methoxyphenol 3a

In the large scale biotransformation of phenol **3a**, the major metabolite, cyclohex-2-en-1-one *cis*-diol **6a** (45 g, 38%

yield), was isolated by crystallization from the crude extract.[5e] GC-MS analysis of the retained combined fractions showed that catechol **13a** and hydroquinone **14a** were also present among a mixture of unidentified metabolites. One very minor metabolite was identified as 2-hydroxy-6-methoxy-6-oxohexanoic acid **15a** by LC-TOFMS analysis: $[M+H]^+$ 177.0758, calcd. for $C_7H_{13}O_5$ 177.0758; $[M+Na]^+$ 199.0577, calcd. for $C_7H_{12}O_5Na$ 199.0582; $[M+NH_4]^+$ 194.1018, calcd. for $C_7H_{16}NO_5$ 194.1028. LC-TOFMS analysis of the combined fractions indicated that another unidentified metabolite was present; its molecular weight was consistent with structure **19a*S***. Separation of the combined fractions by careful column chromatography (hexane →50% EtOAc in hexane) gave a pure sample of metabolite **19a*S***.

(4*S*)-4-Hydroxy-3-methoxycyclohex-2-en-1-one 19a*S*,[14] Colourless oil (1.2 g, 1%); R_f 0.15 (50% EtOAc in hexane); $[\alpha]_D$ - 31.9 (*c* 1.0, $CHCl_3$); HRMS: (TOF-LCMS) $[M+H]^+$ 143.0709, calcd. for $C_7H_{11}O_3$ 143.0708; ^{1}H NMR (400 MHz) δ = 2.01 (1 H, ddddd, *J* =12.5, 10.1, 7.9, 4.3, 0.6 Hz, H-5), 2.25-2.37 (2 H, m, H-5', H-6), 2.59 (1 H, m, H-6'), 2.65 (1 H, br s, OH), 3.76 (3 H, s, Me), 4.47 (1 H, dd, *J* =8.4, 5.0 Hz, H-4), 5.34 (1 H, s, H-2), ^{13}C NMR (100 MHz) δ = 29.5, 34.0, 56.2, 65.8, 102.1, 176.2, 198.6; IR (film) ν_{max}/cm^{-1} 3389, 2945, 1630, 1609, 1231.

(b) Chemoenzymatic synthesis of (*S*)-4-hydroxy-3-methoxycyclohex-2-en-1-one 19a*S*

(*S*)-2-Bromo-2-cyclohexen-1-ol 21*S*. This compound was available as a colourless oil from an earlier biotransformation [5h] of 2-bromo-2-cyclohex-2-en-1-one **20**, $[\alpha]_D$ - 80.3 (*c* 1.77, $CHCl_3$), *ee* 99.8% (chiral GC analysis).

(*S*)-(2-Bromocyclohex-2-enyloxy)-*tert*-butyldimethylsilane 22*S*.[15] *tert*-Butyldimethylsilyl trifluoromethanesulfonate (280 µl, 1.21 mmol) was added to a solution of alcohol **21*S*** (200 mg, 1.10 mmol), maintained at 0°C in dry CH_2Cl_2 (10 mL) containing triethylamine (240 µl, 1.69 mmol). The reaction mixture was stirred (2 h) at 0°C, allowed to warm to room temperature and then ice (20 g) and CH_2Cl_2 (20 mL) were added to it. After thoroughly mixing the reaction mixture by shaking, the organic layer was separated, washed with brine (15 mL), dried (Na_2SO_4), and concentrated to give a yellow oil. It was purified by flash chromatography (hexane) to yield the TBS ether **22*S*** as a colourless oil (310 mg, 94%); R_f 0.4 (hexane); $[\alpha]_D$ - 82.9 (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz) δ = 0.11 (3 H, s, SiMe), 0.17 (3 H, s, SiMe), 0.92 (9 H, s, CMe_3), 1.40 (1 H, m, H-5), 1.55-1.68 (3 H, m, H-5', H-6, H-6'), 1.83 (1 H, m, H-4), 1.95 (1 H, m, H-4'), 4.02 (1 H, m, H-1), 5.97 (1 H, dd, *J* = 4.7, 3.7 Hz, H-3); ^{13}C NMR (100 MHz,) δ = - 4.5, - 4.3, 17.4, 18.3, 26.0 (3C), 27.9, 33.9, 70.8, 126.0, 132.1; IR (film): ν_{max}/cm^{-1} 2949, 2930, 1644, 1252, 1093.

(*S*)-3-Bromo-4-(*tert*-butyldimethylsilyloxy)cyclohex-2-en-1-one 23*S*. A solution of *tert*-butyl hydroperoxide in water (6 M, 0.63 mL, 3.78 mmol) was added dropwise into a mixture of TBS ether **22*S*** (200 mg, 0.68 mmol), K_2CO_3 (47 mg, 0.34 mmol), diacetoxyiodobenzene (670 mg, 2.1 mmol) and butyl butyrate (1.5 mL) maintained at 0°C. The reaction mixture was stirred (0°C, 8 h), diluted with a mixture of 20% ether in hexane (10 mL), filtered (diatomaceous earth), and the filtrate concentrated to give a crude yellow oil. It was purified by column chromatography (5% Et_2O in hexane) to give enone **23*S*** as a colourless oil (60 mg, 29%); R_f 0.28 (5% Et_2O in hexane); $[\alpha]_D$ + 41.5, (*c* 0.5, $CHCl_3$); HRMS: (LC-TOFMS) $[M+H]^+$ 305.0560, calcd. for $C_{12}H_{22}O_2SiBr$ 305.05670; ^{1}H NMR (400 MHz) δ = 0.15 (3 H, s, SiMe), 0.20 (3 H, s, SiMe), 0.93 (9 H, s, CMe_3), 2.07 (1 H, dddd, *J* = 13.7, 7.8, 6.0, 4.6 Hz, H-5), 2.25 (1 H, dddd, *J* = 13.7, 9.1, 4.6, 4.0, H-5' Hz), 2.38 (1 H, ddd, *J* = 16.9, 7.8, 4.6 Hz, H-6), 2.67 (1 H, ddd, *J* = 16.9, 9.2, 4.6 Hz, H-6'), 4.50 (1 H, dd, *J* = 6.0, 4.0 Hz, H-4), 6.44 (1 H, s, H-2); ^{13}C NMR (100 MHz) δ = - 4.6, - 4.4, 18.3, 25.8 (3C), 32.0, 33.4, 71.0, 132.8, 152.6, 196.0; IR (film) ν_{max}/cm^{-1} 2954, 1689, 1610, 1471.

(*S*)-4-(*tert*-Butyldimethylsilyloxy)-3-methoxycyclohex-2-en-1-one 24*S*. To a solution of enone **23*S*** (40 mg, 0.13 mmol) in MeOH (2 mL) was added K_2CO_3 (35 mg, 0.26 mmol), and the mixture kept at room temperature (2 h) without stirring. The reaction mixture was filtered, the filtrate carefully concentrated *in vacuo*, and the light yellow volatile oil obtained was purified by PLC (10% Et_2O in pentane) to furnish methoxy compound **24*S*** as a colourless oil (23 mg, 69%); R_f 0.2 (5% Et_2O in pentane); HRMS: (ES) $[M+H]^+$ 257.1571, calcd. for $C_{13}H_{25}O_3Si$ 257.1573; ^{1}H NMR (400 MHz) δ = 0.08 (3 H, s, SiMe), 0.11 (3 H, s, SiMe), 0.89 (9 H, s, CMe_3), 1.95-2.14 (2 H, m, H-5, H-5'), 2.29 (1 H, ddd, *J* = 16.7, 5.8, 4.4 Hz, H-6), 2.67 (1 H, ddd, *J* = 16.7, 10.1, 5.0 Hz, H-6'), 3.70 (3 H, s, OMe), 4.32 (1 H, dd, *J* = 5.0, 4.1 Hz, H-4), 5.27 (1 H, s, H-2); ^{13}C NMR (100 MHz) δ = - 5.0, - 4.6, 18.3, 25.8 (3C), 31.0, 32.9, 55.8, 67.2, 102.2, 176.9, 199.3; LRMS: (EI): 217 (100), 257 (5), 304 (60), 445 (50), 1065 (15); IR (film) ν_{max} /cm^{-1} 2955, 2930, 1670, 1618, 1226, 834.

(*S*)-4-Hydroxy-3-methoxycyclohex-2-en-1-one 19a*S*. A THF solution of tetrabutylammonium fluoride (1 M, 118 µl, 0.12 mmol) was added to a solution of compound **24*S*** (20 mg, 0.08 mmol) in THF (4 mL) at 0°C. The reaction mixture was stirred and allowed to warm slowly (2 h) to 10°C. The concentrated reaction mixture was purified by PLC (50 % EtOAc in hexane) to give hydroxyenone **19a*S*** as a colourless oil (7 mg, 62%); R_f 0.15 (50% EtOAc in hexane); $[\alpha]_D$ - 48.8 (*c* 0.53, $CHCl_3$). The synthetic sample of hydroxyenone **19a*S*** was found to be identical with the enzymatically formed metabolite **19a*S***.

(c) New metabolites of 2-methoxyphenol 4a

LC-TOFMS analysis of the aqueous bio-extract, obtained from the biotransformation of 2-methoxyphenol substrate **4a**, showed the presence following metabolites: cyclohexanone *cis*-diols **17a*cis*** and **17a*trans***, cyclohex-2-en-1-one *cis* diol **8a**, hydroquinone **16a**, catechol **13a** and its hydroxycarboxylic acid derivative **15a**, and some unidentified bioproducts.[5e] The large-scale biotransformation of 2-methoxyphenol **4a** and purification by column chromatography (hexane → EtOAc) had yielded (2*S*, 3*S*, 4*S*)-*cis*-diol **17a*cis*** (2.5 g) and (2*R*, 3*S*, 4*S*)-*trans*-diol **17a*trans*** (0.25 g).[5e] LC-TOFMS analysis of unidentified pooled chromatography fractions (2.6 g, eluent 50% EtOAc

in hexane), indicated it to be a mixture (9:1) of two isomeric compounds ([M+H]$^+$ 143), which could not be separated. This retained concentrated mixture was re-examined during the study. A pure sample of the major isomer, separated by multiple elution PLC (2.5% MeOH in $CHCl_3$), was identified as 4-hydroxy-2-methoxycyclohex-2-en-1-one **25a*S***. The minor isomer of the mixture was found to be indistinguishable from metabolite 4-hydroxy-3-methoxycyclohex-2-en-1-one **19a*S***, which was derived from 3-methoxyphenol **3a**.

(4*S*)-4-Hydroxy-2-methoxycyclohex-2-en-1-one 25a*S*. Light yellow oil, R_f 0.26 (5% MeOH in $CHCl_3$); $[\alpha]_D$ - 29.0 (*c* 1.1, $CHCl_3$); HRMS: (LC-TOFMS) [M+H]$^+$ 143.0703, calcd. for $C_7H_{11}O_3$ 143.0708; [M+Na]$^+$ 165.05216, calcd. for $C_7H_{10}O_3Na$ 165.0528; [M+K]$^+$ 181.0261, calcd. for $C_7H_{10}O_3K$ 181.0267; [M+H - H_2O]$^+$ 125.0523, calcd. for $C_7H_8O_2$ 125.0524; ^{1}H NMR (400 MHz) δ = 1.94 (1 H, dddd, *J* = 12.6, 11.0, 8.1, 4.5 Hz, H-5), 2.28 (1 H, m, H-5'), 2.40 (1 H, ddd, *J* = 17.0, 11.1, 4.6 Hz, H-6), 2.56 (1 H, bs, OH), 2.68 (1 H, ddd, *J* = 17.0, 6.3, 4.5 Hz, H-6'), 3.60 (3 H, s, OMe), 4.68 (1 H, m, H-4), 5.82 (1 H, dd, *J* = 3.5 Hz, 0.6, H-3); ^{13}C NMR (100 MHz) δ = 32.6, 35.2, 55.5, 66.5, 118.8, 151.4, 194.0. Addition of (2*S*, 3*S*, 4*S*)-3,4-dihydroxy-2-methoxycyclohexanone **17a*cis*** as substrate to *P. putida* UV4, resulted in the formation of metabolite 4-hydroxy-2-methoxycyclohex-2-enone **25a*S*** and provided confirmation of its (4*S*) absolute configuration by stereochemical correlation.

(d) New metabolites of 2-methylphenol (*o*-cresol) 4b

Biotransformation (*P.putida* UV4) of *o*-cresol **4b** was found to yield (4*S*,5*R*,6*S*)-4,5-dihydroxy-6-methylcyclohex-2-en-1-one **8b** as the only identified metabolite.[5a] Repeat biotransformation of phenol **4b**, under similar conditions, and GC-MS analysis of a small portion of the crude concentrate, after extraction with EtOAc and silylation, showed that catechol **13b** and hydroquinone **16b** were present among the minor metabolites. LC-TOFMS analysis (4.31 min.) and comparison with an authentic sample, confirmed that metabolite **8b** was the main component: [M+H]$^+$ 143.0702, calcd. for $C_7H_{11}O_3$ 143.0708 and [M+Na]$^+$ 165.0516, calcd. for $C_7H_{10}O_3Na$ 165.0528. LC-TOFMS analysis also indicated the presence of 4-hydroxy-3-methylcyclohex-2-en-1-one **25b*S*** (11.98 min) and cyclohexanone *cis*-diol **17b*cis*** (3.9 min.) as minor metabolites. PLC purification (50% EtOAc in hexane) yielded 4-hydroxy-3-methylcyclohex-2-en-1-one **25b*S***; the structure and absolute configuration were established by comparison with the literature data of its opposite enantiomer.

(4*S*)-Hydroxy-2-methylcyclohex-2-en-1-one 25b*S*. Light yellow oil (23 mg, 0.56 % yield); R_f 0.20 (50% EtOAc in hexane); $[\alpha]_D$ - 49.0 (*c* 0.7, $CHCl_3$), (Lit.[7] Ent. $[\alpha]_D$ + 46.7, $CHCl_3$); HRMS: (EI) M$^+$ 126.0685, calcd. for $C_7H_{10}O_2$ 126.0681; ^{1}H NMR (400 MHz) δ = 1.79 (3 H, s, Me), 1.95 (1 H, m, H-5), 2.37 (2 H, m, H-4, H-5), 2.62 (1 H, m, H-6), 2.8 (1 H, br s, OH), 4.55 (1 H, m, H-4), 6.73 (1 H, m, H-3); ^{13}C NMR (100 MHz) δ = 15.9, 33.1, 35.9, 66.9, 135.9, 148.4, 199.9.

(2*S*,3*R*,4*S*)-3,4-Dihydroxy-2-methylcyclohexanone 17b*cis*. A solution of 2-methylcyclohex-2-en-1-one *cis*-diol **8b** (5 mg), in methanol (1 mL) containing 10% Pd/C (*ca*. 1 mg), was stirred overnight at room temperature, under hydrogen atmosphere and normal pressure. The catalyst was filtered off, the filtrate concentrated and the product purified by PLC (80% EtOAc in hexane), to give the hydrogenated cyclohexanone *cis*-diol **17b*cis*.** (4.2 mg) as a colourless oil; R_f 0.32 (75% EtOAc in hexane); $[\alpha]_D$ + 1.2 (*c* 0.35, MeOH); LC-TOFMS: 3.9 min. [M+H]$^+$ 145.0859, calcd. for $C_7H_{13}O_3$ 145.0865; [M+NH_4]$^+$ 162.1125, calcd. for $C_7H_{16}NO_3$ 162.1130; [M+Na]$^+$ 167.0679, calcd. for $C_7H_{12}O_3Na$ 167.0684; ^{1}H NMR (400 MHz) δ = 1.14 (1 H, d, *J* = 6.9 Hz, Me), 1.63 (2 H, br s, 2x OH), 2.03-2.20 (2 H, m, H-5, H-5'), 2.33-2.41 (2 H, m, H-6, H-6'), 2.57 (1 H, qd, *J* = 6.9, 2.6 Hz, H-2), 4.14 (1 H, m, H-4), 4.17 (1 H, ddd, *J* = 11.0, 5.3, 2.6 Hz, H-3); ^{13}C NMR (100 MHz) δ = 10.8, 28.4, 38.0, 47.0, 70.8, 76.9, 209.8. The sample of compound **17b*cis*** obtained by hydrogenation showed identical LC-TOFMS data to that of metabolite **17b*cis***.

(e) GC-MS analysis of diTMS derivatives of 4-hydroxycyclohex-2-en-1-ones 19e*R* and 19a*S* and triTMS derivatives of phenol hydrates (cyclohexa-1,5-diene-1,4-diols) 19e'*R* and 19a'*S*

Trimethylsilylation of metabolites **19e*R*** and **19a*S*** with MSTFA, and GC-MS analyses of the silyl derivatives, showed two peaks in each case. The major peaks were due to the monoTMS derivatives of the keto tautomers **19e*R*** (5.05 min., 88%, [M+H]$^+$, *m/z* = 184) and **19a*S*** (8.61 min., 81%, [M+H]$^+$, *m/z* = 214). The minor peaks (6.40 min., 12%, [M+H]$^+$, *m/z* = 256) and (8.69 min., 19%, [M+H]$^+$, *m/z* = 286) were consistent with triTMS derivatives of the corresponding enol tautomers **19e'*R*** and **19a'*S***.

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Enzyme-catalysed synthesis of cyclohex-2-en-1-one *cis*-diols from substituted phenols, anilines and derived 4-hydroxycyclohex-2-en-1-ones

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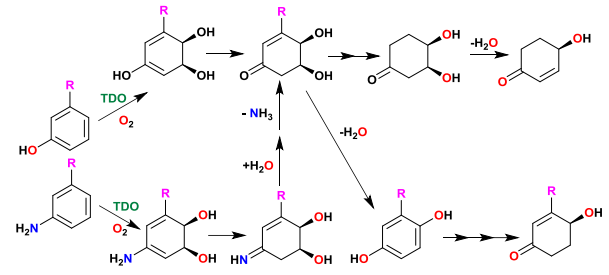

- Whole cell biotransformations of anilines and phenols usinig *Pseudomonas putida* UV4
- New *cis*-diol metabolites from toluene dioxygenase (TDO) catalysed dihydroxylation of anilines
- New hydroxycyclohexenone metabolites from phenol and hydroquinone substrates